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# Horizontal spread of *Rhodococcus equi* macrolide resistance plasmid pRErm46 across environmental *Actinobacteria*

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## 1 ABSTRACT

2       Conjugation is one of the main mechanisms involved in the spread and maintenance of  
3 antibiotic resistance in bacterial populations. We recently showed that the emerging macrolide  
4 resistance in the soil-borne equine and zoonotic pathogen *Rhodococcus equi* is conferred by the  
5 *erm*(46) gene carried on the 87-kb conjugative plasmid pRErm46. Here we investigated the  
6 conjugal transferability of pRErm46 to 14 representative bacteria likely encountered by *R. equi*  
7 in the environmental habitat. *In vitro* mating experiments demonstrated conjugation to different  
8 members of the genus *Rhodococcus* as well as to *Nocardia* and *Arthrobacter* spp. at frequencies  
9 ranging from  $\sim 10^{-2}$  to  $10^{-6}$ . pRErm46 transfer was also observed in mating experiments in soil  
10 and horse manure, albeit at a low frequency and after prolonged incubation at 22-30°C  
11 (environmental temperatures), not 37°C. All transconjugants were able to transfer pRErm46 back  
12 to *R. equi*. Conjugation could not be detected with *Mycobacterium* and *Corynebacterium* spp. or  
13 several members of the more distant phylum *Firmicutes* such as *Enterococcus*, *Streptococcus* or  
14 *Staphylococcus*. Thus, pRErm46 host range appears to span several actinobacterial orders with  
15 certain host restriction within the *Corynebacteriales*. All bacterial species that acquired  
16 pRErm46 expressed increased macrolide resistance with no significant deleterious impact on  
17 fitness, except in the case of *Rhodococcus rhodnii*. Our results indicate that actinobacterial  
18 members of the environmental microbiota can both acquire and transmit the *R. equi* pRErm46  
19 plasmid and thus potentially contribute to the maintenance and spread of *erm*(46)-mediated  
20 macrolide resistance in equine farms.

## 21    **IMPORTANCE**

22            This study demonstrates the efficient horizontal transfer of the *R. equi* conjugative  
23    plasmid pRErm46, recently identified as the cause of the emerging macrolide resistance among  
24    equine isolates of this pathogen, to and from different environmental *Actinobacteria* including a  
25    variety of rhodocci as well as *Nocardia* and *Arhtrobacter* spp. The reported data support the  
26    notion that environmental microbiota may act as reservoirs for the endemic maintenance of  
27    antimicrobial resistance in an antibiotic pressurized farm habitat.

## INTRODUCTION

*Rhodococcus* spp. are present in diverse environments owing to their unique capacity for niche adaptation [1-3]. Much of their environmental plasticity relies on extrachromosomal genetic elements of circular or linear topology that carry key niche-adaptive traits[4, 5]. While environmental *Rhodococcus* spp. typically harbor plasmids encoding catabolic pathways[3, 6], *R. equi*, the only animal pathogen of this genus, carries the pVAP virulence plasmids essential for pathogenesis and survival in host macrophages[5, 7]. Three pVAP plasmid types have been described so far, each adapted to a specific animal host: the equine-associated pVAPA, porcine-associated pVAPB and ruminant-associated pVAPN[8-10]. Although *R. equi* can infect a variety of animal species including humans, young foals are particularly susceptible and develop a severe respiratory disease characterized by focal purulent bronchopneumonia[11, 12].

*R. equi* is a ubiquitous soil organism that becomes endemic in horse breeding farms where it causes high morbidity and mortality in foals [12, 13]. Due to the lack of an effective vaccine and the insidious nature of the initial stages of the infection, many farms rely on thoracic ultrasonographic screening followed by antibiotic treatment of foals presenting subclinical lung lesions[11]. Field studies indicate, however, that this practice may constitute an example of unjustified antibiotic misuse because many subclinically infected foals would spontaneously recover independently of antibiotic prophylaxis [14, 15]. Not surprisingly, the inception of the mass antibioprophylaxis in 2001 has resulted a few years later in the emergence of resistance to the antimicrobials used in such treatments, a combination of a macrolide and rifampin [13, 16-18]. The emergence of this dual resistance is problematic because only a few antimicrobials are clinically effective to combat rhodococcal foal pneumonia and the macrolide-rifampin combination remains the mainstay of antimicrobial therapy against equine *R. equi*

infection [19]. The macrolide-rifampin resistance emerged upon horizontal transfer of a novel rRNA methylase gene, *erm*(46), which confers resistance to macrolides, lincosamides and streptogramin B (MLS<sub>B</sub>) [20], to a specific *R. equi* strain carrying a novel chromosomal *rpoB* mutation (S531F), which then gave rise to a clonal population [21].

We recently reported that *erm*(46) is acquired and mobilized as part of a conjugative plasmid, pRErm46 [20, 21]. This macrolide resistance plasmid has so far only been identified in equine (pVAPA positive) *R. equi* isolates, likely as a result of the selective pressure exerted by the mass antimicrobial treatments systematically applied to control foal pneumonia in endemic farms. Whether pRErm46 can also be maintained and spread by *R. equi* bacteria carrying the porcine-associated (pVAPB) or ruminant-associated (pVAPN) virulence plasmid types is not known. Homology analysis of the coding sequences of pRErm46's replicon indicated an actinobacterial origin, particularly rhodococcal, but whether pRErm46 can actually be mobilized and replicate in other *Actinobacteria* remains also to be determined. The purpose of this study was to explore the host range of pRErm46 and elucidate whether other members of the environmental microbiota can act as its reservoir in the absence of *R. equi*, thus potentially contributing to the maintenance and perpetuation of the macrolide resistance determinant in the farm habitat.

## RESULTS

### 1. pRErm46 can be self-transferred to and maintained by different actinobacterial species

In a first series of experiments, mating assays were carried out using macrolide-resistant strain 103<sup>+</sup>Apra<sup>R</sup> (pRErm46) as a donor and porcine clinical isolate REPB1 Rmp<sup>R</sup> (pVAPB positive) and bovine isolate REPN1 Rmp<sup>R</sup> (pVAPN-positive) as recipient strains (see Table 6).

pRErm46 was mobilized to the *R. equi* porcine and bovine isolates at conjugation frequencies of  $\sim 10^{-5}$  and  $\sim 10^{-4}$  respectively (Table 1). The presence of the *R. equi* virulence plasmids (pVAPB and pVAPN) and macrolide resistance plasmid pRErm46 was verified by PCR in the dual resistant (to erythromycin [Erm<sup>R</sup>] and rifampin [Rmp<sup>R</sup>]) transconjugant colonies. The porcine isolate transconjugants were confirmed to still carry pVAPB in addition to the pRErm46 macrolide resistance plasmid. However, none of the bovine isolates that received pRErm46 kept the pVAPN virulence plasmid. Plasmid incompatibilities are based on similarities in the origin of replication of the amplicons, whereby competition for replication factors favors plasmids which, due e.g. to smaller size (as would be the case of the 87-kb pRErm46 vs the 120-kb pVAPN), have a faster replication [22, 23]. We searched the pVAPN putative origin of replication sequence 5'-AAAACCCCCAGGTGGGGGTGGGTTTT [9] in the pRErm46 DNA sequence using Blast and we identified a 33-nt segment (5- AAAACCCCCAGCCATGCGGGGCT GAGGGTTTCT) upstream the open reading frame (ORF) PRERM\_0270 (23985-24018 bp) that shared 25 of the 27 nt of pVAPN's sequence (Figure S1). No such sequence was identified in the replicon of the pVAPA/B plasmids.

We next explored the host range of pRErm46 by performing bacterial conjugation assays using the same donor and 14 representative bacterial species including six non-*equi* *Rhodococcus* spp. (*Rhodococcus defluvii* DSM45893, *Rhodococcus fascians* DSM20669, *Rhodococcus rhodochrous* JCM2156, *Rhodococcus erythropolis* JCM2892, *Rhodococcus ruber* JCM3205, *Rhodococcus rhodnii* JCM3203); five different *Actinobacteria* (*Corynebacterium pseudotuberculosis*, *Mycobacterium smegmatis* MKD8, *Mycobacterium fortuitum* and *Nocardia globerula* ATCC21505 from the Order *Corynebacteriales*, *Arthrobacter paraffineus* ATCC19958 from the Order *Micrococcales*); and three species from the more distant Phylum

*Firmicutes* (*Enterococcus faecalis* ATCC29212, an equine field *Streptococcus zooepidermicus* subsp. *equi* isolate and *Staphylococcus aureus* ATCC29213). All *Rhodococcus* species successfully acquired pRErm46 at varying transfer frequencies (Table 1). Similarly, pRErm46 was conjugally transferred to *N. goberula* and *A. paraffineus* at comparable ratios to *Rhodococcus* spp. (Table 1). In contrast, transfer of pRErm46 could not be detected to the tested mycobacteria, *C. pseudotuberculosis* and the non-actinobacterial species. pRErm46 transconjugants were subsequently used as donors in conjugation assays with *R. equi* recipients. Notably, all primary recipients of pRErm46 were able to mobilize the plasmid back to macrolide susceptible *R. equi* at similar transfer frequencies (Table 1).

The macrolide resistance phenotype of five randomly selected transconjugant colonies per recipient bacteria from the *in vitro* mating assays with the *R. equi* 103<sup>+</sup>Apra<sup>R</sup> (pRErm46) donor (see above) was evaluated by susceptibility testing using eTest strips. Minimum inhibitory concentrations (MIC) to macrolides were tested before and after acquisition of pRErm46. The MIC of recipients prior and after gaining pRErm46 were 0.5-8 µg/ml and ≥ 256 µg/ml, respectively, for all isolates. The only exception was *R. fascians* in which the MIC increased from 0.19-0.38 µg/ml to 8 -12 µg/ml (Table 2).

## **2. Transfer of pRErm46 in soil and manure**

To assess whether pRErm46 transfer can take place in conditions approximating the equine farm habitat, bacterial mating experiments were performed in soil and horse manure. Macrolide and rifampin resistant equine clinical isolate PAM2287[21] (prototype strain of the pRErm46-harboring *R. equi* clone) was used as pRErm46 donor and susceptible (pRErm46-negative) avirulent *R. equi* 103<sup>-</sup> with an apramycin resistance *aac(3)IV* cassette [103<sup>-</sup>Apra<sup>R</sup>][24]



as the recipient in the mating assay, enabling transconjugant selection via dual resistance to erythromycin (Erm<sup>R</sup>) and apramycin (Apra<sup>R</sup>). Three different conjugation ratios (1:1, 1:10 and 10:1), and four different temperatures (4°C, 22°C, 30°C and 37°C) to mimic seasonal temperature changes, were tested. Soil and manure samples were screened for conjugation at 7, 30, 90 and 180 days. pRErm46 transfer in both soil and horse manure was sporadically observed after 30 days incubation at 22°C and 30°C, independently of the donor:recipient ratio (Table 3). Transconjugants were detected in all cases, although mostly in only one of the triplicate soil samples per time point and tested temperature. Calculated transfer ratios ranged from 10<sup>-6</sup> to 10<sup>-2</sup> transconjugants/recipient bacteria. For confirmation, the presence of the Apra<sup>R</sup> cassette, *erm*(46) gene, pRErm46, in addition to the virulence plasmid pVAPA, was tested by PCR in up to 10 (depending on transconjugant numbers) Ery<sup>R</sup> and Apra<sup>R</sup> colonies. All transconjugants screened by PCR carried the the *aac*(3)*IV* (Apra<sup>R</sup>) cassette and the *erm*(46) (Erm<sup>R</sup>) gene, providing molecular confirmation of the transconjugant phenotype. pRErm46 was detected by PCR in all transconjugants tested except for the those isolated after 90 days at 30°C with a 10:1 donor:recipient ratio, despite these testing positive for the *erm*(46) gene. Interestingly, these transconjugants are those in which pVAPA acquisition had been detected in our experiments. Based on these data, optimal environmental mating conditions appear to be 22°C-30°C for a length of between 8 to 30 days.

Under the above optimal conditions for pRErm46 environmental conjugation, we used the same assay to test the mobilization of pRErm46 from *R. equi* to *R. erythropolis* (as a representative of the *Rhodococcus* genus), *N. globerula* and *A. paraffineus* in soil (Table 4). The three species successfully acquired pRErm46 after 15 days. All soil samples tested contained transconjugant bacteria, with *N. globerula* showing the highest transfer ratio ranging from 10<sup>-1</sup> to

10<sup>-4</sup> transconjugants/recipient bacteria. The mobilization of pRErm46 to *R. erythropolis* occurred at similar frequencies to those observed for *R. equi* in soil and ranged between 10<sup>-5</sup> and 10<sup>-7</sup> transconjugants/recipient bacteria, while slightly lower transfer ratios were observed using *A. paraffineus* as recipient (10<sup>-6</sup>-10<sup>-9</sup> transconjugants/recipient).

### 3. pRErm46 fitness cost varies in different recipients.

To gain further insight into the determinants of pRErm46 maintenance, we measured the impact of pRErm46-mediated macrolide resistance on bacterial fitness. *R. erythropolis*, *A. paraffineus* and *N. globerula* showed no significant differences in exponential growth rate and maximum growth upon acquisition of pRErm46 neither in complex medium (BHI) nor *R. equi* chemically defined medium (mREMM) (Table 5, Fig. 1). Surprisingly, some of the tested strains (*R. fascians* and *R. rhodnii*) even showed significantly improved fitness in mREMM medium when carrying pRErm46 (Table 5). This was particularly evident with *R. fascians* which failed to grow in mREMM in the absence, but not presence, of pRErm46, suggesting the intriguing possibility that some plasmid-encoded determinants may contribute to regulate bacterial growth in nutrient-limiting conditions. The only detrimental effect of pRErm46 on bacterial fitness was observed with *R. rhodnii*, which when harboring pRErm46 manifested slightly impaired growth (Table 5, Fig 5).

## DISCUSSION

Horse breeding farms that use macrolides and rifampin as a mass treatment for *R. equi* subclinical pneumonia likely represent highly antibiotic-pressurized environments [17, 25], where bacterial survival is contingent upon acquisition of a suitable resistant phenotype. This

study explores how the horizontal transfer dynamics of the *R. equi* MLS<sub>B</sub> resistance plasmid pRErm46 to indigenous members of the environmental microbiota may contribute to the maintenance of a resistant microbial pool in equine farms. pRErm46 was successfully mobilized *in vitro* to six different *Rhodococcus* spp. as well as to *N. globerula*. This was unsurprising as most of the genes of the pRErm46 genetic backbone have an obvious rhodococcal origin[21] and because phylogenetic evidence supports that *Nocardia* is the closest *Corynebacteriales* genus to *Rhodococcus* [26, 27]. The fact that no conjugal transfer could be detected with *Firmicutes* species nor representative species of other *Corynebacteriales* such as *Corynebacterium* or *Mycobacterium* while it was observed with the *Actinomycetales* species *A. paraffineus* suggests that pRErm46's host range is essentially actinobacterial while at the same time exhibits genus-specific restrictions. Further work is required with additional representative bacteria commonly found in the equine farm habitat or equine microbiome to more specifically delineate the conjugal transfer range of the *R. equi* macrolide resistance determinant and the potential mechanisms underpinning its persistence in equine farms.

*erm(46)*-driven macrolide resistance has only been reported in *R. equi* equine isolates carrying the pVAPA virulence plasmid [21]. This study shows that pRErm46 can be also acquired by *R. equi* carrying the pVAPB porcine plasmid, thus indicating that porcine environments/isolates may also theoretically contribute to the spread and maintenance of *erm(46)*-mediated *R. equi* macrolide resistance. This might not to be the case for ruminant-associated *R. equi* isolates based on the observed potential incompatibility of the pRErm46 and pVAPN replicons. Our findings with the pVAPN plasmid imply that differences in compatibility among plasmids, typically abundant among soil-dwelling *Actinobacteria*, may be a critical factor in shaping pRErm46's host range.

189           Despite the ease with which pRErm46 is conjugally transferred between *R. equi* isolates  
190 [21, 28], and to other rhodococci or other susceptible *Actinobacteria* as shown here, the MLS<sub>B</sub>  
191 resistance determinant *erm*(46) has until now only been found in a specific *R. equi* clonal  
192 population[21]. This may be explained by the requirement of a strong antibiotic selective  
193 pressure for the maintenance of pRErm46[21], apart from the fact that no systematic searches  
194 have been undertaken to detect pRErm46 in the environmental microbiome. In addition, our *in*  
195 *vitro* data may not be an accurate reflection of what occurs in the farm environment. To  
196 approximate such conditions, we carried out mating assays in soil and horse manure, at four  
197 different incubation temperatures to mimic seasonal changes. Despite using a large conjugation  
198 mix of  $\sim 10^7$  CFU/g of soil (100 times higher than the concentrations at which *R. equi* is typically  
199 found in soil in endemic farms [16]), conjugation remained sporadic but consistently detectable  
200 after 8 to 30 days at temperatures of 22-30°C, independently of the donor:recipient ratio used.  
201 These results demonstrate that conjugal transfer of the *R. equi* macrolide resistance plasmid can  
202 occur in the equine farm environment, potentially contributing to its spread and endemicity.

203           Interestingly, in the soil experiments we noted that all transconjugant colonies that  
204 resulted from the 10:1 (donor:recipient) matings after a 90 day incubation at 30°C had acquired  
205 both the *erm*(46) gene and the pVAPA virulence amplicon while pRErm46's transfer was not  
206 detected. *erm*(46) is actually carried within a highly mobile transposon (TnRErm46) harbored by  
207 pRErm46, from which we previously found it readily transposes to the *R. equi* genome including  
208 the virulence plasmid[21]. We therefore assume that the observed pRErm46-independent transfer  
209 of *erm*(46)-mediated macrolide resistance reflects the co-option of the transfer functions of an  
210 indigenous mobile element (most likely pVAPA, but potentially other extrachromosomal  
211 elements present in the microbiota present in the soil sample tested as well). This finding

highlights the extraordinary horizontal spread potential of the *erm*(46) determinant via the transposition functions of the highly mobile TnRErm46 element[21].

Finally, we explored potential bacterial fitness costs associated with the acquisition of the pRErm46 plasmid by components of the environmental microbiota. Similar to what we had previously observed in *R. equi* [21], pRErm46 showed a neutral effect on fitness *in vitro*, or even promoted higher growth rates than the corresponding isogenic strains lacking the plasmid, in all bacteria species tested excepting *R. rhodnii*. Although *in vitro* fitness assays may not accurately reflect the growth dynamics of bacteria in soil and manure, our results indicate that under suitable conditions environmental bacteria could serve as a potential reservoir for pRErm46 and, hence, MLS<sub>B</sub> resistance, in the absence of the primary host organism *R. equi* (and even antibiotic selective pressure).

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 6. *R. equi* isolates were routinely cultured in Brain-Heart Infusion medium (BHI, Difco Laboratories-BD) at 30°C, 200 rpm, unless otherwise stated. Agar media were prepared by adding 1.6 % of bacteriological agar (Oxoid). Media were supplemented with antibiotics (erythromycin 10 µg/ml, rifampin 100 µg/ml, apramycin 50 µg/ml; Sigma) whenever required.

**Rifampin resistant strain derivation.** Prior to the mating experiments, rifampin resistant ( $Rmp^R$ ) derivatives of recipient species were obtained as previously described [9] for selection of transconjugants by double antimicrobial resistance ( $Erm^R$  and rifampin  $Rmp^R$ ). Briefly, several well-isolated colonies were collected, resuspended in PBS and streaked onto a BHI plate supplemented with 25 µg/ml of rifampin. After incubation for 48 h at 30°C, the  $Rmp^R$  phenotype was selected and stabilized by restreaking a few colonies in a fresh BHI plate supplemented with 100 µg/ml rifampin.

**In vitro bacterial conjugation assay.** Conjugation assays were carried out as described in Anastasi et al. 2015[28]. *R. equi* donor and recipient strains were grown overnight in BHI (in the presence of antibiotic when required), washed twice with PBS and adjusted to  $OD_{600} = 1$ . Then, 100 µl of donor suspension were mixed with 100 µl of the recipient 1:1 in a microtube. The mixture was centrifuged (6000 rpm, 10 min), resuspended in 5 µl of sterile BHI and spotted in a thick drop onto a BHI plate. After 72 h incubation at 30°C, the bacterial mixture was scraped and resuspended in PBS, and serial dilutions plated onto BHI agar supplemented with rifampin (recipient selection) or rifampin plus erythromycin (transconjugant selection). Transconjugants

were confirmed by PCR. Conjugation ratios were calculated using the following formula:

Conjugation ratio = n. of transconjugant cell/ n. recipient cells

**Conjugation assay in soil and horse manure.** Bacterial mating assays were performed in soil and horse manure (collected from University of Georgia teaching farm) in parallel. For each time point and condition, 3g of soil/manure were placed in three 5ml test tubes (1gr/tube) and inoculated with  $10^7$  CFU/g containing 10:1, 1:1 or 1:10 donor:recipient ratios. Then, soil/manure was stirred during 30 min for an even bacterial distribution and incubated at four different temperatures 4°, 22°, 30° and 37°C for up to 180 days. The presence of macrolide mobilization was checked at 5 time points: Day 0 [control], 7, 30, 90 and 180. For each time point and condition, 3 g of soil/manure (coming from 3 independent test tubes) were quantitatively cultured by serial 10-fold dilutions on *R. equi* selective NANAT[29] supplemented with corresponding antibiotics for transconjugant and recipient bacteria selection. < 10 transconjugant colonies were confirmed by PCR (S2). Conjugation ratios were calculated as stated above.

**Polymerase chain reaction.** PCRs were carried out using C100 thermal cycler (Bio-Rad) and GoTaq® Flexi DNA Polymerase (Promega) following general parameters.

**DNA sequencing and analysis:** Sanger sequencing was performed by Eurofins (Louisville, Kentucky). Sequences were analyzed using Ape plasmid editor (Wayne Davis) and the Basic Local Alignment Search Tool (BLAST [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) was used for the alignment of DNA sequences against the reference sequence.

**Antimicrobial susceptibility testing.** Bacteria were prepared from overnight cultures in tryptic soy agar (TSA) by the direct colony suspension method according to the guidelines established by the CLSI, resulting in the recommended inoculum of  $\sim 1$  to  $5 \times 10^5$  CFU as verified by colony counting. The MICs of erythromycin were determined by use of ETEST® strips (bioMerieux).

**Bacterial growth assays.** Potential bacterial fitness costs associated with the acquisition of the pRErm46 were investigated by monitoring the growth rate of macrolide -susceptible and -resistant isogenic strains (that received pRErm46 by conjugation) in complex media BHI and in chemically defined medium mREMM[21],24. Bacteria were grown overnight in BHI, washed twice with PBS and adjusted to  $OD_{600} = 1$ . Then, 400  $\mu$ l/well of bacteria in the selected media were added in triplicate to 48-well plates (Corning). The assays were conducted using an automated plate reader (Synergy HT, BioTeK) at 37° C, at 200rpm. Measurements were taken every 30 min. Data were analyzed using BioTeK Gen5 Data Analysis Software (BioTeK). Data were processed by biological growth curve fitting package “growthrates” [30] in statistical software R (version 3.6.1). Growth parameters (i) exponential growth and (ii) maximal bacterial yield were analyzed for significant differences using paired t-test also in R (version 3.6.1).

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292

293 **AUTHOR CONTRIBUTIONS**

294 Álvarez–Narváez, S.: Manuscript writing, experimental design, data analysis and research  
295 implementation.

296 Giguere, S.: Experimental design and data analysis.

297 Berghaus, L.J.: Antimicrobial susceptibility testing, help in manuscript preparation.

298 Dailey C.: Data analysis.

299 Vázquez-Boland J.A.: Experimental design, conceptualization, data analysis and manuscript  
300 writing.

301

302 **CONFLICT OF INTEREST**

303 The authors declare that the research was conducted in the absence of any commercial or  
304 financial relationships that could be construed as a potential conflict of interest.

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**TABLE 1.** *R. equi* pRErm46 heterologous conjugation experiments.

Donor	Recipient	Transfer frequency <sup>a</sup>
<i>R. equi</i> 103S (pRErm46)	<i>R. equi</i> porcine isolate (pVAPB)	$9.14 \pm 6.98 \times 10^{-5}$
	<i>R. equi</i> bovine isolate (pVAPN)	$4.41 \pm 1.42 \times 10^{-4}$
	<i>R. defluvi</i>	$1.62 \pm .85 \times 10^{-5}$
	<i>R. erythropolis</i>	$2.49 \pm 1.12 \times 10^{-5}$
	<i>R. rhodochrous</i>	$4.31 \pm 2.91 \times 10^{-2}$
	<i>R. rhodnii</i>	$7.51 \pm 6.94 \times 10^{-4}$
	<i>R. ruber</i>	$3.39 \pm 1.55 \times 10^{-5}$
	<i>R. fascians</i>	$1.6 \pm .28 \times 10^{-7}$
	<i>N. globerula</i>	$7.9 \pm 5.8 \times 10^{-3}$
	<i>A. paraffineus</i>	$5.1 \pm 3.8 \times 10^{-5}$
	<i>M. smegmatis</i>	$< 10^{-10}$
	<i>M. fortuitum</i>	$< 10^{-10}$
	<i>C. pseudotuberculosis</i>	$< 10^{-10}$
	<i>S. aureus</i>	$< 10^{-10}$
	<i>S. zooepidemicus</i>	$< 10^{-10}$
	<i>E. faecalis</i>	$< 10^{-10}$
<i>R. equi</i> porcine isolate (pVAPB, pRErm46)	Susceptible <i>R. equi</i> 103 <sup>c</sup>	$4.09 \pm 2.37 \times 10^{-6}$
<i>R. equi</i> bovine isolate (pRErm46)		$1.46 \pm .62 \times 10^{-5}$
<i>R. defluvi</i> (pRErm46)		$8.47 \pm 4.60 \times 10^{-6}$
<i>R. erythropolis</i> (pRErm46)		$1.95 \pm 1.12 \times 10^{-5}$
<i>R. rhodochrous</i> (pRErm46)		$5.97 \pm 2.85 \times 10^{-5}$
<i>R. rhodnii</i> (pRErm46)		$2.71 \pm 2.21 \times 10^{-5}$
<i>R. ruber</i> (pRErm46)		$2.68 \pm 2.67 \times 10^{-6}$
<i>R. fascians</i> (pRErm46)		$1.11 \pm .39 \times 10^{-7}$
<i>N. globerula</i> (pRErm46)		$1.18 \pm .66 \times 10^{-5}$
<i>A. paraffineus</i> (RErm46)		$1.23 \pm 1.22 \times 10^{-5}$

<sup>a</sup> Number of transconjugant/recipient CFU. Data represent means  $\pm$  SD ( $n = 3$  experiments).

**TABLE 2.** Erythromycin MICs of different bacteria upon *erm*(46) conjugal acquisition. Data refers to MIC in mg/L determined using Etest ( $n = 3$ ).

Species	<i>erm</i> (46) negative	<i>erm</i> (46) positive
<i>R. equi</i> porcine isolate	0.25 - 0.5	>256
<i>R. equi</i> bovine isolate	0.5	>256
<i>R. defluvii</i>	6 - 8	>256
<i>R. erythropolis</i>	0.25 - 0.38	>256
<i>R. fascians</i>	0.19 - 0.38	8 - 12
<i>R. rhodnii</i>	0.38 - 0.75	>256
<i>R. rhodochrous</i>	3 - 4	>256
<i>R. ruber</i>	0.64	>256
<i>A. paraffineus</i>	0.50 - 0.75	>256
<i>N. globerula</i>	0.38 - 1	>256

**TABLE 3.** Conjugal transfer of pRErm46 in soil and horse manure.

Sample	Ratio <sup>a</sup>	Temperature	day 30			day 90			day 180		
			sample1	sample2	sample3	sample1	sample2	sample3	sample1	sample2	sample3
Soil	1:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	$2.56 \times 10^{-4}$	-	-	-	-
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	1:10	4°C	-	-	-	-	-	-	-	-	-
		22°C	$9.43 \times 10^{-6}$	$1.58 \times 10^{-6}$	$2.38 \times 10^{-5}$	-	-	-	-	-	$1.14 \times 10^{-4}$
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	10:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	$6.25 \times 10^{-4}$	-	-
		30°C	-	-	-	-	-	$*5.10 \times 10^{-2}$	$1.14 \times 10^{-2}$	-	-
		37°C	-	-	-	-	-	-	-	-	-
Manure	1:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	$2.11 \times 10^{-2}$	-	-	-	-	-	-	-
		30°C	-	$2.21 \times 10^{-5}$	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	1:10	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	-	-	-
		30°C	-	-	-	-	$1.25 \times 10^{-5}$	-	-	$2.21 \times 10^{-5}$	-
		37°C	-	-	-	-	-	-	-	-	-
	10:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	-	-	-
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-

Data represents conjugation transfer frequencies per sample of soil/horse manure. 3 samples were collected each time point and incubation temperature.

<sup>a</sup> Ratio refers to the ratio donor:recipient bacteria used in each conjugation assay

**TABLE 4.** pRErm46 transfer from *R. equi* to other species in soil.

Species	Ratio <sup>a</sup>	Temperature	day 15	day 30
<i>R. erythropolis</i>	1:1	22°C	$2.43 \pm 2.02 \times 10^{-5}$	$3.32 \pm 1.99 \times 10^{-5}$
		30°C	$9.66 \pm 5.69 \times 10^{-6}$	$3.05 \pm 1.46 \times 10^{-6}$
	1:10	22°C	$1.24 \pm .40 \times 10^{-6}$	$1.29 \pm .97 \times 10^{-7}$
		30°C	$1.91 \pm 1.06 \times 10^{-5}$	$6.29 \pm 2.20 \times 10^{-6}$
<i>A. paraffineus</i>	1:1	22°C	$9.98 \pm 9.98 \times 10^{-9}$	$1.66 \pm .98 \times 10^{-7}$
		30°C	$2.78 \pm 1.15 \times 10^{-6}$	$2.18 \pm 1.55 \times 10^{-6}$
	1:10	22°C	$5.61 \pm 6.47 \times 10^{-8}$	$3.63 \pm 1.98 \times 10^{-7}$
		30°C	$4.94 \pm 2.39 \times 10^{-7}$	$9.07 \pm 6.67 \times 10^{-7}$
<i>N. globerula</i>	1:1	22°C	$1.39 \pm .81 \times 10^{-1}$	$2.32 \pm 2.07 \times 10^{-2}$
		30°C	$5.62 \pm 2.22 \times 10^{-1}$	$0.97 \pm 1.19 \times 10^{-3}$
	1:10	22°C	$9.38 \pm 8.25 \times 10^{-3}$	$4.82 \pm 2.37 \times 10^{-4}$
		30°C	$6.06 \pm 3.47 \times 10^{-2}$	$8.33 \pm 8.33 \times 10^{-3}$

Data represent means  $\pm$  SDs of 3 samples collected per time point and incubation temperature.

<sup>a</sup> Ratio refers to the ratio donor:recipient bacteria used in each conjugation assay.

**TABLE 5.** Growth data of isogenic bacteria in the presence and absence of the macrolide resistance plasmid pRErm46 in BHI and chemically defined medium. The maximum growth rate during exponential growth and maximal bacterial growth during the growth curve were estimated from fits of the OD600 values using Growrates package in R. Asterisk represents significant ( $P<0.05$ ) increase based on paired t-test statistical analysis (n=3).

**(A) BHI**

	Maximum Exponential Growth Rate ( $\text{h}^{-1}$ )		Maximal Bacterial Growth (OD600)	
	pRErm46	No pRErm46 -	pRErm46	No pRErm46
<i>R. erythropolis</i>	0.24 ( $\pm$ .007)	0.24 ( $\pm$ .009)	1.60 ( $\pm$ .05)	1.59 ( $\pm$ .05)
<i>R. fascians</i>	0.17 ( $\pm$ .02)	0.21 ( $\pm$ .02)*	0.71 ( $\pm$ .20)	0.63 ( $\pm$ .14)
<i>R. rhodnii</i>	0.13 ( $\pm$ .002)*	0.11 ( $\pm$ .004)	0.69 ( $\pm$ .03)	1.07 ( $\pm$ .03)*
<i>A. paraffineus</i>	0.26( $\pm$ .06)	0.26 ( $\pm$ .02)	1.35 ( $\pm$ .03)	1.42 ( $\pm$ .03)
<i>N. globerula</i>	0.12 ( $\pm$ .01)	0.14 ( $\pm$ .02)	2.07 ( $\pm$ .29)	2.04 ( $\pm$ .28)

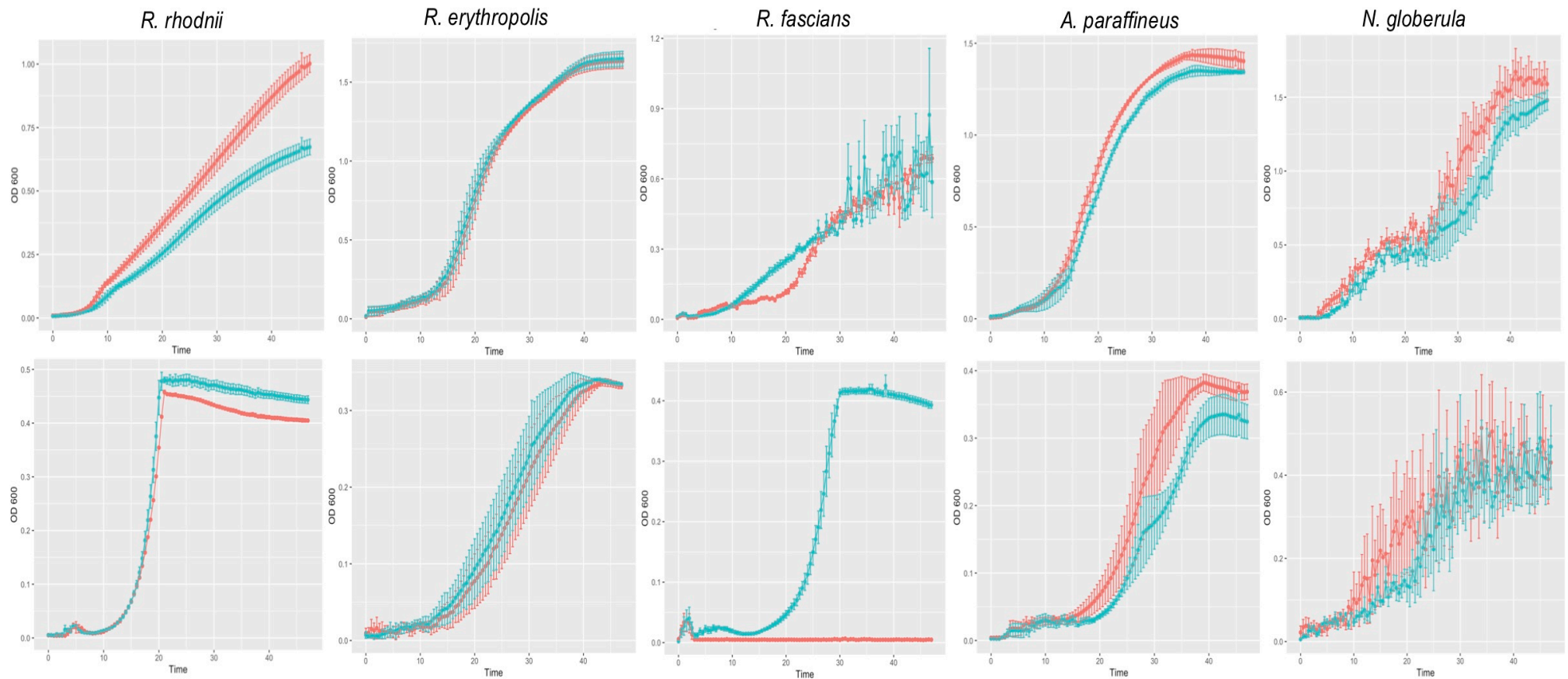
**(B) mREMM**

	Maximum Exponential Growth Rate ( $\text{h}^{-1}$ )		Maximal Bacterial Growth (OD600)	
	pRErm46	No pRErm46	pRErm46	No pRErm46
<i>R. erythropolis</i>	0.22 ( $\pm$ .02)	0.22 ( $\pm$ .02)	0.36( $\pm$ .006)	0.36 ( $\pm$ .01)
<i>R. fascians</i>	0.39 ( $\pm$ .12)*	0.005 ( $\pm$ .004)	0.54 ( $\pm$ .06)*	0.02 ( $\pm$ .02)
<i>R. rhodnii</i>	0.96 ( $\pm$ .01)*	0.86 ( $\pm$ .003)	0.46( $\pm$ .008)*	0.42 ( $\pm$ .003)
<i>A. paraffineus</i>	0.46( $\pm$ .21)	0.41 ( $\pm$ .07)	0.36 ( $\pm$ .04)	0.38 ( $\pm$ .01)
<i>N. globerula</i>	0.23 ( $\pm$ .06)	0.26 ( $\pm$ .06)	0.43 ( $\pm$ .05)	0.44 ( $\pm$ .05)



**TABLE 6.** Bacterial strains used in this study.

Species	Description	Source
<i>Rhodococcus equi</i>		
103 <sup>-</sup> ApraR	Plasmidless 103 strain containing the <i>aac(3)IV</i> apramycin resistance gene integrated on the chromosome	Tripathi <i>et al.</i> 2012
PAM2287	Macrolide and Rifampin clinical isolate	Alvarez <i>et al.</i> 2019
103 <sup>+</sup> ApraR, pRERM46	Derivative strain from 103- ApraR. pRERM46 and pVAPA plasmids introduced by conjugal transfer	Giguère collection
REPB1	Rifampin resistant derivative strain from porcine clinical isolate	This study
REPN1	Rifampin resistant derivative strain from bovine clinical isolate	This study
<i>Rhodococcus</i> spp		
<i>R. defluvi</i> RmpR	Rifampin resistant derivative strain from <i>R. defluvi</i> DSM45893	This study
<i>R. erythropolis</i> RmpR	Rifampin resistant derivative strain from <i>R. erythropolis</i> JCM 2892	This study
<i>R. fascians</i> RmpR	Rifampin resistant derivative strain from <i>R. fascians</i> DSM20669	This study
<i>R. rhodnii</i> RmpR	Rifampin resistant derivative strain from <i>R. rhodnii</i> JCM 3203	This study
<i>R. rhodochrous</i> RmpR	Rifampin resistant derivative strain from <i>R. rhodochrous</i> JCM 2156	This study
<i>R. ruber</i> RmpR	Rifampin resistant derivative strain from <i>R. ruber</i> JCM 3205	This study
Other bacteria		
<i>Nocardia globerula</i> RmpR	Rifampin resistant derivative strain from <i>N. globerula</i> ATCC 21505	This study
<i>Arthrobacter paraffineus</i> RmpR	Rifampin resistant derivative strain from <i>A. paraffineus</i> ATCC19958	This study
<i>Mycobacterium smegmatis</i> RmpR	Rifampin resistant derivative strain from <i>M. smegmatis</i> MKD8	This study
<i>Mycobacterium fortuitum</i> RmpR	Rifampin resistant derivative strain from <i>M. fortuitum</i> from Hondalus strain collection	This study
<i>Staphylococcus aureus</i> RmpR	Rifampin resistant derivative strain from <i>S. aureus</i> ATCC 29213	This study
<i>Enterococcus faecalis</i> RmpR	Rifampin resistant derivative strain from <i>E. faecalis</i> ATCC 29212	This study
<i>Streptococcus equi</i> subspecies <i>zooepidemicus</i> RmpR	Rifampin resistant derivative strain from <i>S. zooepidemicus</i> wild-type clinical isolate (from Giguère strain collection)	This study



**Figure 1. Acquisition of pRErm46 has a different fitness cost depending on the recipient species. pRErm46 was conjugally transferred from *R. equi* to (i) *R. rhodnii*, (ii) *R. erythropolis*, (iii) *R. fascians* (iv) *A. paraffineus* and (v) *N. globerula*. Growth curves with each isogenic set composed by a pRErm46+ (red) and a pRErm46- (turquoise) isolate in rich complex medium (BHI) and chemically defined medium (mREMM, see Materials and Methods).**